A QUANTITATIVE STUDY OF THE INFLUENCE OF CERTAIN NEUTRAL SALTS UPON THE ACTIVITY OF MALT AMYLASE

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DISSERTATION

Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in the Faculty of Pure Science,
Columbia University.

BY
MARION CLEAVELAND

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Introduction

For many years a relation between enzymic activity and the presence of neutral salts has been recognized. In 1875 Nasse[1] from his work with crude salivary and pancreatic amylases as well as with diastase stated that these enzymes depended upon the presence of neutral salts in a specific way. Nevertheless many contradictory statements may be found concerning the manner and extent to which malt amylase is affected by neutral salts.

Among the factors contributing to this lack of agreement may be ascribed various differences in the experimental conditions. The presence of salts and other foreign materials in crude malt extract doubtless has been the cause of conflicting data. Differences in the purity of the constituents of the substrate, such as starch and salts, may easily account for discrepancies, since the utmost purity of materials is required for accurate work with such sensitive entities as enzymes. The method of determining diastatic power may also influence the experimental results. The want of a fixed ratio between the amyloclastic and saccharogenic powers of malt amylase has already indicated that the iodine color reaction is not always adequate as a means of following the hydrolysis of starch.[2] The action of the amylase depends also upon such conditions as time, temperature, hydrogen ion activity and the concentrations of the constituents of the substrate.

Inasmuch as the importance of these factors of enzymic activity has been realized only with the continuous development of the study of enzymes, the earliest investigations involving the relation of neutral salts to the amylase of malted barley have little but historical interest. As early as 1845 Bourchadat[3] noted that the chlorides,

sulfates, and acetates of certain alkali and alkaline earth metals did not hinder the action of diastase upon gelatinized starch. Later Dumas[4] found that dilute malt was rendered inactive by borax. In 1886 Hirschfeld[5] found extraordinary saccharifying ability in a saturated sodium chloride solution of a preparation made from malt by a combination of lead acetate and alcohol precipitations. Moritz[6] also reported a favorable influence of sodium chloride upon malt but found little or no effect due to sodium nitrate or to sodium, calcium or magnesium sulfates.

A new period of amylase investigation began in 1886 with Lintner[7] who not only instituted the use of soluble starch, a substrate permitting more uniform results, but who also studied suitable methods of separating and purifying the active starch-splitting constituent of malt extract. Lintner found that the chlorides of sodium, potassium and calcium in concentrations above 0.4% favored the formation of sugar from starch by purified preparations of malt amylase. This favorable effect of neutral salts, usually sodium chloride, has been noted also by Gruss[8] and Moraczewski[9] with diastase and by Osborne and Campell[10] and Wroblewski[11] with their purified malt amylase preparations. Effront,[12] however, asserted that the favorable action of sodium chloride upon diastase existed no longer when chemically pure sodium chloride was used. Similar contradictions appeared in the literature about ten years after this when the effect of neutral salts upon commercial diastase or malt extract was reported to be favorable by Ford,[13] Schneidewind,[14] and Hawkins[15] or to be negligible according to Fernbach and Wolff[16].

In 1907 Preti[17] claimed that dialysis did not weaken the amylase of malt but Frankel and Hamburg[18] in 1906 found the enzyme to be more sensitive the greater its purity. In 1912 Lisbonne and Vulquin[19] reported that the complete removal of mineral matter from malt extract resulted in a totally inactivated solution, the enzymic

activity of which was restored in part by the addition of such neutral salts as the alkali chlorides. The purification method consisted in prolonged dialysis against conductivity water followed by prolonged electrodialysis. This process occupied from two to three weeks in which time it is probable that the amylase itself suffered hydrolytic destruction to some extent. Fricke and Kaja[20] in 1924 resorted to both electrodialysis and electroosmosis in order to purify malt amylase. Their preparations were more active than the original diastase and were not influenced by the addition of neutral salts although the ash content had decreased greatly during the process.

Sherman and Thomas[21] investigated the influence of certain acids and salts upon purified preparations of malt amylase. The saccharogenic power of the enzyme when acting upon two percent dispersions of purified and neutralized starch was increased by the addition of thrice crystallized chloride, nitrate or sulfate of sodium and potassium. The increased activity varied from two to three times that obtaining in the absence of neutral salt; the optimal salt concentration varied from 0.1 to 0.5 molar. The amyloclastic action of malt amylase as measured by the Wohlgemuth method reached an optimum at much lower salt concentrations than those required for optimal saccharogenic activity. Although the amounts of various acids and acid salts necessary for the optimal saccharogenic activity of malt amylase were found to give the substrate the same actual acidity, the relation of the effect of different hydrogen ion activities and different neutral salt concentrations was not studied. In 1917 Thomas[22] found that the recrystallized bromides of sodium and potassium inhibited the activity of malt amylase when the molar salt concentration was less than 0.1 but at higher concentration they resembled the other neutral salts previously investigated.

Hahn and Harpuder[23] have studied in some detail the influence of neutral salts upon the amyloclastic action of salivary and malt amylases by means of the Wohlgemuth

iodine method. Both the starch and the commercial diastase were dialysed but no reference was made to the purity of the other materials such as the neutral salts themselves or the water used to make the substrate mixture. The influence of neutral salt at a known hydrogen ion activity as compared with that of a buffer salt alone was found to depend upon the concentrations of the buffer and neutral salt and upon the reaction of the system. Sodium and potassium chloride, bromide, sulfate and nitrate and also ammonium chloride caused dialysed malt extract to be less active on the acid side of the optimum with higher concentrations of acetate than in the presence of the same concentration of acetate alone. When the acetate concentration was lowered (N/300), these same salts at the same concentration and hydrogen ion activity increased the enzymic activity above that exerted in the presence of N/300 acetate. On the alkaline side of the optimum the increased activity of malt amylase diminished with increasing concentrations of salt and buffer, but only in the case of sulfate was the enzymic activity retarded. Thus it was found that the acitivity of malt amylase could be increased by a certain salt concentration above that exerted in the presence of buffer salt alone; in the more acid substrates this increased enzymic activity was apparent only with lower concentrations of acetate. These investigators also established the fact that malt amylase differed from salivary in that its optimum was not changed upon the addition of neutral salts.

Hahn and Harpuder found that sodium salts exerted a greater influence than potassium salts in changing the isoelectric point of malt amylase to more acid values. Since these neutral salts appeared to effect a change in the charge on the enzyme but did not influence the activity optimum, the authors concluded that there is little if any connection between the electrical behavior of malt amylase and its enzymic activity. The fact that solutions of commercial malt diastase were used may explain why their values of the isoelectric point and the activity opti-

mum did not coincide, as has been established by Sherman, Thomas and Caldwell[24] with purified preparations of malt amylase.

The combined precipitating effect of neutral salts and acetate has also been studied under Hahn[25,26] upon solutions of commercial diastase. A certain critical buffer concentration was found at which the addition of neutral salt caused no change in the opalescence or precipitation due to the acetate alone. When the acetate concentration was greater or less than the critical value, the effect of neutral salt was to increase or decrease the precipitation due to acetate alone. This critical acetate concentration was smaller the more acid the reaction. Due to the general similarity of these results to those obtained in the same laboratory in regard to the influence of neutral salts upon the activity of dialysed malt extract, Hahn concluded that enzymic activation resulted from the increased surface concomitant with decreased size of amylase particles and that the growth of the active particles accounted for retardation of enzymic activity. The fact that the enzyme concentration varied three hundred fold in the activity and precipitation experiments and that the character of the precipitate obtained from the crude diastase was unknown was realized by the authors to weaken the force of their hypothesis.

Euler and Svanberg[27] found only a very small increase in the saccharogenic power of dialysed malt extract due to the presence of sodium chloride in 0.24 and 0.47 molar concentration with the substrate adjusted with phosphate. The fact that no change in enzymic activity was noticeable in the presence of the chloride, sulfate and nitrate of potassium led to the conclusion that the decrease in activity accompanying the purification process could not be ascribed to the removal of these salts.

According to Ernstrom[28] the addition of sodium chloride even in five percent concentration did not affect the activity of malt extract purified by prolonged dialysis. The substrate comprised one percent starch adjusted by

means of phosphate to pH=5. The maltose formed was determined according to Bertrand.

Neither iodide nor fluoride ions were found by Olsson[29] to affect the amount of sugar formed by dialysed malt extract. Olsson attributed cases of inactivation by iodide to traces of free iodine. Holmberg[30] found 0.01N potassium iodide sufficient to completely poison malt amylase at pH=5.1. That this effect may have been due to free iodine is probable, for Pincussen[31] found that the increased production of free iodine in potassium iodide solutions under the influence of light was paralled by the increased inactivation of malt amylase by light in solutions of this same salt.

Hirzume[32] found that neither halogen salts nor serum increase the ability of malt extract to liquefy starch and that these reagents seemed to increase the susceptibility of the enzyme to destruction by heat.

Recently Pincussen[33] has found that salts of the alkali and alkaline earth metals increase the susceptibility of dialysed malt extract to destruction by ultraviolet light and that the order in which the ions act is practically the opposite of that for the heat inactivation of the enzyme.

This review of the literature reveals little that is consistent even in the most recent data dealing with the influence of neutral salts upon the activity of malt amylase. Indeed with the exception of the contributions from this laboratory[21,22] and the extensive studies of Hahn and co-workers,[23,25,26] the subject has been investigated only incidentally. Although Hahn realized the necessity of employing purified amylase and starch and the important interrelation of the conditions of salt and buffer concentrations and hydrogen ion activity in their effect upon the enzymic activity, yet the results are open to criticism in that apparently no precautions were exercised in regard to the purity of the salts used and also in that the starch hydrolysis was followed by the less satisfactory Wohlgemuth method.

Recently the influence of neutral salts upon the activ-

ity of pancreatic amylase has been investigated quantitatively by Sherman, Caldwell and Adams.[34] The interrelation of hydrogen ion activity, adjusted by 0.01 molar phosphate, and of salt concentration was studied and then comparisons were made of the influence of the different salts, each at its optimal concentration and hydrogen ion activity, upon the action of pancreatic amylase. All the materials for the investigation were carefully purified. The optimal hydrogen ion activity for pancreatic amylase was found to depend upon the kind and concentration of the neutral salt. A decrease in the optimal hydrogen ion activity occurred with increasing salt concentration up to a certain point, after which, increase in concentration of neutral salt had no appreciable influence upon the optimum. It was also established that "the salts which are less favorable to the activity of pancreatic amylase are required in higher concentration and in turn exert their most favorable influence in solutions of higher hydrogen ion activities than do those salts which are more efficient activators." The influence of neutral salts appeared to be specific and to be due mainly to the anions. Phosphate and sulfate were found to have no effect. The other salts investigated could be arranged in the following order according to the decreasing magnitude of their influence upon the hydrolysis of starch by pancreatic amylase: sodium and potassium chlorides, lithium chloride, sodium bromide, sodium nitrate, sodium chlorate, sodium sulfocyanate, sodium fluoride.

Differences in the action of the starch-splitting enzymes of plant and animal origin have long been recognized. The fact that malt amylase is relatively stable in aqueous solution, not requiring the presence of electrolytes that it may show enzymic activity, is a noteworthy way in which it differs from the animal amylases. Since there has been no such extensive quantitative investigation of the relation of neutral salts to the amylase of germinated grains as that conducted with pancreatic amylase, it appeared that such a study might lead to a better understanding of

the nature of the amylases as well as to the practical usefulness of establishing the optimal conditions for the activity of malt amylase. The following investigation was therefore undertaken.

The experimental work was devised with these questions in mind:

(1) Does sodium chloride either favor or inhibit the activity of malt amylase and how is this relationship, if any, affected by changes in the hydrogen ion activities and sodium chloride concentrations of the system?

(2) Is the effect of neutral salt upon the activity of malt amylase due to the anion or to the cation? The influence of potassium chloride in comparison with that of sodium chloride was investigated to establish this point. In view of the tremendous and apparently equal activation of the animal amylases by sodium and potassium chlorides and of the many differences between the amylases of different origin, it seemed possible that potassium salts might be preferable for the activity of the plant amylases.

(3) Does any series of anions similar to that which has been found for pancreatic amylase[34] exist in regard to their influence upon the activity of malt amylase? In addition to the chlorides of sodium and potassium, the sulfate and nitrate of sodium were studied to illustrate the influence of a divalent and of another univalent anion upon the enzymic activity.

Thus the determination of the interrelation of the influences of hydrogen ion activity and neutral salts and of changes in their concentrations upon the activity of malt amylase was the purpose of the following study.

Experimental

Materials. A preparation of malt amylase was made according to a modification of the method of Sherman and Schlesinger.[35] Malt extract which had been dialysed against distilled water and finally against triply distilled water for about thirty hours was precipitated fraction-

ally with alcohol. The 50%–70% precipitate when dried yielded a preparation the activity of which was 400 on the scale of Sherman, Kendall and Clark.[36] For purposes of comparison two other preparations made by Sherman and Schlesinger about fifteen years ago were used. These older preparations had lost about four-fifths of their original high powers and in general gave less smooth results.

Soluble starch prepared according to Lintner was repeatedly washed by decantation first with distilled water and then with triply distilled water. During the washing process the starch was kept in the ice-box to avoid fermentation. The starch was then air-dried with due precautions against access of dust and the percentage of moisture was determined.

In general the salts which were of the highest purity obtainable were purified further by two recrystallizations from distilled water and one recrystallization from triply distilled water. In the case of sodium chloride some difficulties were encountered with samples that had been purified by double precipitation with hydrogen chloride and ignition in platinum. Therefore a method for purifying sodium chloride outlined by Richards and Wells was adopted.[37] C. P. hydrochloric acid was diluted to about 20% hydrogen chloride and redistilled after a preliminary heating with a few crystals of potassium permanganate to remove traces of bromides and iodides. This redistilled hydrochloric acid was neutralized by means of solid C. P. sodium bicarbonate. From the resulting solution sodium chloride was precipitated with hydrogen chloride, obtained by warming concentrated C. P. hydrochloric acid and thoroughly washing the gas by bubbling it through four wash bottles containing either C. P. hydrochloric acid or triply distilled water. A second precipitation and three recrystallizations served to purify the sodium chloride further. It was then filtered by suction and dried over night at 100°C. Great care was observed in the above purification processes to avoid contamination from dust. Also, rubber connections were eliminated in the

hydrogen chloride gas train. The glassware and filter paper were rinsed with redistilled hydrochloric acid and distilled water. Triply distilled water was always used for the solutions and the final rinsing of glassware. Ignition in platinum was avoided due to the liability of contamination by impurities difficult to remove from platinum surfaces or by materials coming from the walls of the muffle furnace. An extremely rigorous removal of hydrogen chloride from the sodium chloride was not attempted since its presence would not materially change the hydrogen ion activity of the buffered substrate for enzymic action.

Acetic acid "special" procured from J. T. Baker was used with no further treatment.

To obtain triply distilled water for the preparation of the digestion mixtures, for the enzyme solutions and for the final rinsing of all glassware, ordinary distilled water was first distilled from alkaline permanganate through glass and then from a very dilute phosphoric acid solution through block tin. It was kept in bottles of special resistant glass.

Method. The general procedure employed in this investigation consisted in allowing the enzyme to act at 40°C± 0.01 for thirty minutes upon two percent starch dispersions which differed among themselves with respect to either the salt content or the hydrogen ion activity or both. At the expiration of this half-hour period the extent to which the starch had been converted to reducing sugars (mainly maltose)[38] was established according to the gravimetric Fehling method generally followed in this laboratory.[39]

The hydrogen ion activities of the substrates were fixed by 0.01 molar acetate mixtures. The choice of this salt depended upon the work of Kintzi[40] who pointed out that acetate mixtures whose maximum buffer effect occurs at those hydrogen ion activities most favorable to the activity of malt amylase were more desirable than phosphate mixtures. This investigator found a slight activation of

malt amylase at 40°C. by both phosphate and acetate, but that the very slight influence exerted by acetate was complete at 0.0001 molar concentration whereas that exerted by phosphate was still noticeable in concentrations as high as 0.01 molar. In the present work a concentration of acetate was required which would be sufficient to afford both the desired buffer action and a safe margin above the amount needed to completely activate the enzyme but which would at the same time be low enough to avoid any impairment to the influence of the neutral salts to be studied. The concentration selected to fulfill these conditions was 0.01 molar. The greater sensitivity of the enzyme to differences in hydrogen ion activity at this low acetate concentration as found by Kintzi[40] was thought not undesirable in the investigation to be undertaken upon the combined effects of neutral salts and hydrogen ion activity upon malt amylase.

Thus the fixed experimental conditions existing during the enzymic hydrolysis of starch were time, temperature, and the concentrations of starch, enzyme and total acetate. The separate determinations may be classified according to a very few types. (1) In order to determine whether or not sodium chloride exerted any influence upon the action of malt amylase, direct comparisons were made of the enzymic activity (a) at graded hydrogen ion activities in the presence and absence of the salt at a fixed concentration, and (b) in the presence of graded concentrations of the salt at fixed hydrogen ion activities. (2) The other neutral salts studied, potassium chloride, sodium nitrate and sodium sulfate, were compared in a similar manner as to their effect upon the hydrolysis of starch by malt amylase. The enzymic activity in the presence of these salts at a single concentration and hydrogen ion activity was compared with that exerted in the absence of salt both at the same hydrogen ion activity and at the optimum. (3) Finally the effect of different concentrations of each salt upon the activity of malt amylase was established at a single hydrogen ion activity (a)

at the optimum, (b) on the alkaline side of the optimum, (c) on the acid side of the optimum. Numerous cases in which direct comparisons were made of the effect of different pairs of salts upon the starch hydrolysis under different conditions of salt concentration or hydrogen ion activity substantiate general comparisons between the four salts.

Some details of the determination of amylase activity may be given briefly. The proper amount of air-dried starch was mixed into a paste, poured slowly into hot water and boiled for three minutes to effect a clear dispersion. It was then cooled and diluted to known volume. To separate portions of the starch were added 0.1 molar sodium acetate and 0.1 molar acetic acid in suitable proportions to give upon dilution two percent starch dispersions of the desired hydrogen ion activity as adjusted by 0.01 molar acetate. The neutral salt content was varied at will by the addition of the proper volumes of salt solution to the starch mixtures before the final dilution. One hundred cubic centimeters of the various substrates were then put in a water bath maintained at 40°C±0.01 by a Freas thermostat. A small sample of each starch suspension was set aside in the ice-box for the subsequent measurement of its hydrogen ion activity.

During the half hour allowed for the substrate to attain the proper temperature, a solution of the purified preparation of malt amylase was made by first rubbing ten milligrams of the dry preparation to a paste with a drop or two of water and diluting to 50 cc with ice cold water. One cubic centimeter portions of the enzyme solution were used. The warm starch dispersions were added to the enzyme in order at fifteen second intervals. The starch hydrolysis was allowed to continue at 40°C±0.01 for 30 minutes, whereupon the enzymic action was stopped by the addition of 50 cubic centimeters of freshly mixed Fehling's solution at 15 second intervals in the same sequence as that followed previously in the starch additions. The cuprous oxide formed by the reducing

sugars during a 15 minute period in a boiling water bath was filtered upon Gooch crucibles and was weighed as such. In the following discussion the activity of malt amylase will be expressed as milligrams cuprous oxide.

Great care was exercised in the cleaning of glassware, in the protection of all solutions from dust and in the exclusion of light from the enzyme. All solutions except the Fehling's solution were prepared with fresh, triply distilled water. Bottles of special resistant glass were used for the storing of all solutions.

The hydrogen ion activity of each starch dispersion was measured electrometrically. Readings of the potential difference between a hydrogen electrode and a saturated calomel half cell of the type described by Wilson and Kern[41] in the given starch dispersions were made at room temperature on a Leeds-Northrup portable potentiometer. The hydrogen ion activity was then calculated as pH[42] according to the equation:

$$pH = \log \frac{1}{(H^+)} = \frac{\text{E.M.F. (observed)} - \text{E.M.F. (Calomel half cell)}}{0.000198\ T}$$

Results

Results with Sodium Chloride. The activity of a purified preparation of malt amylase was determined in the presence and absence of 0.01 and 0.02 molar sodium chloride at graded hydrogen ion activities. Typical average results are given in Table I and Figure I.

It is evident that the enzymic activity was higher in the presence of sodium chloride at unfavorable hydrogen ion activities on both sides of the optimum; this effect was measurable in solutions whose hydrogen ion activities were either more acid than a pH value of 4.3 or more alkaline than a pH value of 5.1. When the acidity of the substrate lay between these two pH values, added sodium chloride neither decreased nor increased appreciably the saccharifying ability of the enzyme. Although the enzyme was favored it could not be said to

TABLE I

TYPICAL DATA SHOWING THE INFLUENCE OF SODIUM CHLORIDE AT GRADED HYDROGEN ION ACTIVITIES UPON THE ACTIVITY OF MALT AMYLASE

(a) Influence of 0.01 M NaCl.

	0.01 M NaCl	No NaCl	Increased Activity due to Salt
pH	mg. Cu_2O	mg. Cu_2O	mg. Cu_2O
4.0	261	242	19
4.2	267	262	5
4.3	268	268	0
4.5	264	267	–3
4.7	259	260	–1
4.9	254	255	–1
5.0	249		
5.2		230	
5.5	213	199	14
5.7	210	192	18
6.2	163	150	13

(b) Influence of 0.02 M NaCl.

	0.02 M NaCl	No NaCl	Increased Activity due to Salt
pH	mg. Cu_2O	mg. Cu_2O	mg. Cu_2O
3.9	264	232	32
4.0	266	246	20
4.2	271	261	10
4.3	268	268	0
4.4	269		
4.6		265	
4.7	261	262	–1

In Table I and all the following tables, the activity of malt amylase is expressed in terms of milligrams cuprous oxide.

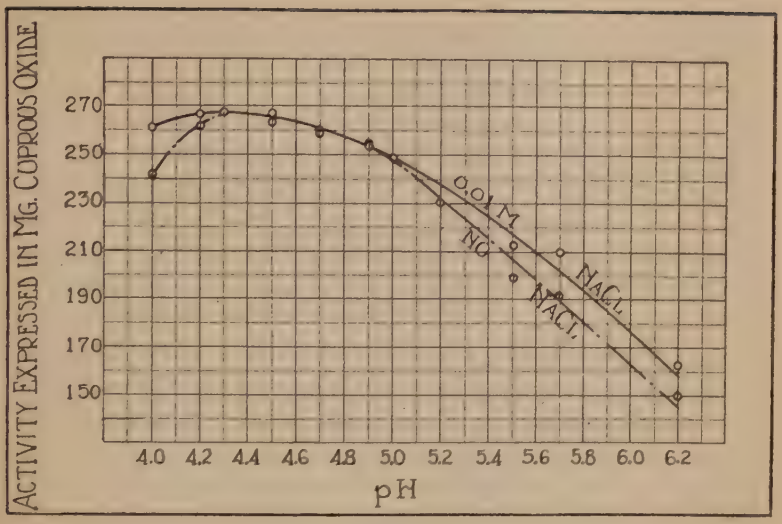

FIGURE I

THE INFLUENCE OF 0.01 MOLAR SODIUM CHLORIDE UPON THE ACTIVITY OF MALT AMYLASE

have been truly activated by sodium chloride, for in no instance was malt amylase rendered more active by the presence of sodium chloride than when acting alone at its optimal hydrogen ion activity.

These findings in regard to the beneficial effect of sodium chloride upon the activity of malt amylase have been confirmed repeatedly. Averages of the differences in enzymic activity caused by the addition of sodium chloride to substrates at hydrogen ion activities on either side of the optimum correspond to an increase of 24 milligrams cuprous oxide in the case of either 0.01 or 0.02 molar salt at a pH value of 4.0, and to increases of 20 and 33 milligrams cuprous oxide in the case of 0.01 and 0.02 molar sodium chloride respectively, at a pH value of 5.5. Five to eight separate determinations have been averaged in the case of 0.01 molar concentration of added salt and three to four in the case of 0.02 molar salt. These data

obtained from separate experiments support the results of the activity curve given above, namely, that in solutions whose hydrogen ion activities are higher or lower than the optimal value, malt amylase is enabled by the addition of sodium chloride to form a larger amount of reducing sugar than in the absence of neutral salt. The lack of any effect at the optimum is also apparent from the small differences in enzymic activity in the presence of 0.01 and 0.02 molar sodium chloride corresponding to 1 and 4 milligrams cuprous oxide respectively.

Due to this beneficial effect of sodium chloride upon malt amylase the enzyme may exert its highest activity in solutions differing from each other more widely in hydrogen ion activity than is possible with only buffered substrate. On the acid side of the optimum where the steepness of the activity curve for malt amylase indicates a very rapid decrease in activity with increasing acidity, 0.01 molar sodium chloride caused a pronounced increase in enzymic activity. On the alkaline side of the optimum, however, where changes in hydrogen ion activity diminish the enzymic power more gradually, the favorable influence of sodium chloride appeared less suddenly. As the accompanying graph (Figure I) shows, malt amylase at 40°C. displays its optimal activity in solutions adjusted to hydrogen ion activities corresponding to values of pH=4.3 to 4.6 when in the absence of sodium chloride; and in solutions of pH values 4.1 to 4.6 when sodium chloride was present in 0.01 molar concentration.

In general, similar results were obtained with the two concentrations of sodium chloride, the influence of which was investigated in this detailed manner upon the action of malt amylase. When the hydrogen ion activity of the solution was especially unfavorable to the enzyme (pH=5.5), 0.02 molar sodium chloride was found to augment the enzymic activity to a greater extent than the lower concentration (0.01M), as was shown by the average differences corresponding to 33 and 20 milligrams

of cuprous oxide respectively. Therefore, that the best conditions for enzyme action might be established, the influence of different concentrations of sodium chloride upon the enzyme was studied further.

A consideration of the results obtained so far made an investigation of the effect of concentration of sodium chloride upon malt amylase at closely graded hydrogen ion activities seem unnecessary. Nevertheless, it did appear to be of value to ascertain how sodium chloride in higher concentrations would influence the enzyme in solutions whose hydrogen ion activities were fixed (a) at the optimum, (b) on the alkaline side of the optimum, (c) on the acid side of the optimum. The pH values of 4.5, 5.5, 4.0 were selected as suitable for this purpose since in solutions corresponding to the second and third of these values malt amylase had been found to be more active in the presence of sodium chloride in 0.01 and 0.02 molar concentration. In spite of the fact obvious from the electrometric determinations of hydrogen ion activities, that 0.01 molar acetate did not buffer the substrate completely against the "neutral salt effect," it was thought preferable to maintain the same low acetate concentration used in the previous determinations that the sensitivity of the enzyme to the salt in question might not be impaired. The very slight changes in enzymic activity with small differences in hydrogen ion activity at the optimum especially and also on the alkaline side of the optimum obviated the necessity of fixing the pH value of the substrate more rigidly.

In solutions of about pH 4.5 in which the enzyme exerts its highest action, the presence of 0.01 to 0.10 molar sodium chloride did not bring about any decided change in the activity of malt amylase. A small increase corresponding to 5 milligrams cuprous oxide may be neglected. The "neutral salt effect" was not a factor here as it was found that the maximum variation in the acidity of these substrates due to the different concentrations of salt added was from pH4.5 to 4.3, while in three of the four

determinations a variation of only 0.1 on the pH scale occurred.

When the substrate was adjusted to values on the alkaline side of the optimum (near pH=5.5) the enzymic activity was increased with each increase in the concentration of sodium chloride up to 0.05 molar above which little if any change in enzymic activity was noted. In the three determinations made, the acidity of the solutions containing the different concentrations of sodium chloride varied in one case by 0.15 on the pH scale and by 0.10 in the other two cases. A comparison of the data with the activity curve of malt amylase showed that the increased activity of the enzyme in 0.05 to 0.10 molar sodium chloride was too great to be due solely to the increase in hydrogen ion activity.

The effect of different concentrations of sodium chloride was next extended to the case of substrates adjusted to a hydrogen ion activity more acid than the optimum. In 0.01 molar sodium chloride solution the enzyme was decidedly more efficient at pH=4.0. An increase in the amount of salt present up to 0.10 molar caused a decrease in the enzymic activity corresponding to 30 milligrams cuprous oxide, which nullified the increased activity evidenced with the lesser concentration of sodium chloride. Since this increase in the concentration of sodium chloride in the substrate was accompanied by a shift in the acidity index from pH 4.0 to 3.9 and inasmuch as the activity curve of malt amylase is very steep on the acid side of the optimum, it was doubtful whether the decrease in enzymic activity with increasing salt concentration was due to the salt itself or to the concomitant change in the acidity. Therefore, the hydrogen ion activities of the starch dispersions were fixed more rigidly by varying the proportions of acetic acid and sodium acetate but always maintaining a total acetate concentration of 0.01 molar.

When the acidity of the substrate was thus kept carefully at pH 4.0 a small decrease in the enzymic activity occurred as the sodium chloride concentration was in-

creased from 0.01 to 0.10 molar. Thus on the acid side of the optimum malt amylase is influenced most favorably by 0.01 molar sodium chloride.

Three typical determinations illustrating the influence of sodium chloride in different concentrations upon malt amylase at the three different hydrogen ion activities investigated are given in Table II. Table III contains a summary of the average differences in enzymic activity due to the presence of sodium chloride at these different concentrations.

TABLE II

INFLUENCE OF THE CONCENTRATION OF SODIUM CHLORIDE UPON THE ACTIVITY OF MALT AMYLASE AT DIFFERENT HYDROGEN ION ACTIVITIES

(Typical Individual Comparisons)

pH	4.0		4.3–4.5		5.4–5.5	
Molar Conc. NaCl	Activity	Increased Activity due to salt	Activity	Increased Activity due to salt	Activity	Increased Activity due to salt
	mg. Cu_2O		mg. Cu_2O		mg. Cu_2O	
0	217		255		206	
0.01	244	27	259	4	231	25
0.02	242	25	258	3	241	35
0.05	237	20	262	7	249	43
0.07	240	23	263	8	252	46
0.10	235	18	260	5	257	51

TABLE III

AVERAGE INCREASES IN THE ACTIVITY OF MALT AMYLASE DUE TO THE PRESENCE OF DIFFERENT CONCENTRATIONS OF SODIUM CHLORIDE AT DIFFERENT HYDROGEN ION ACTIVITIES

Molar Conc. NaCl	0.01	0.02	0.05	0.07	0.10
pH	mg. Cu_2O	mg. Cu_2O	mg. Cu_2O	mg. Cu_2O	mg. Cu_2O
4.0	24	25	21	23	18
4.5	1	6	6	7	5
5.5	19	33	44	46	48

In the early part of this work it was established that sodium chloride in 0.01 and 0.02 molar concentrations does not cause malt amylase to exert higher activity than that exhibited in the absence of the salt at the optimal hydrogen ion activity. The question next arose: Is the activity of malt amylase at its optimal hydrogen ion activity in the absence of salts ever exceeded in solutions of other concentrations of sodium chloride? Since direct comparisons show that the activity of the enzyme at pH 4.0 in the presence of optimal sodium chloride concentration (0.01 molar) is no greater than the highest activity of the amylase in the absence of salts at pH 4.5, this question may be answered in the negative for the case of substrates adjusted to the acid side of the optimum. Moreover a single direct comparison of the concentration effect of sodium chloride upon the activity of malt amylase in solutions adjusted to pH=4.0 and pH=5.5 proved that the enzyme at pH=5.5 in the presence of 0.10 molar sodium chloride (the optimal concentration) possessed the very same activity as that exerted on the acid side of the optimum where only 0.01 molar sodium chloride is required. It might be more valuable to consider average values of all the data for the amylase in the presence and absence of salt at pH values of 4.0, 4.5, and 5.5 which are given in Table IV.

It is interesting to note that 0.01 molar sodium chloride favors the action of malt amylase in solutions on the acid side of the optimum as powerfully as 0.10 molar salt does on the alkaline side. Purified malt amylase even when in the presence of the most desirable concentration of sodium chloride at unfavorable hydrogen ion activities on either side of the optimum does not possess greater activity than that obtaining at its optimum where the presence or absence of neutral salt is immaterial. Malt amylase is not activated by sodium chloride.

Inasmuch as the experimental conditions for amylase investigation have been developed continuously, it appeared interesting to compare the above findings with the

TABLE IV

INFLUENCE OF SODIUM CHLORIDE IN OPTIMAL CONCENTRATION UPON THE ACTIVITY OF MALT AMYLASE REFERRED TO THE ACTIVITY AT pH 4.5 IN THE ABSENCE OF THE SALT

Molar Conc. NaCl	pH	Activity mg. Cu_2O	Percentage
0	4.0	232	89
0.01	4.0	256	99
0	4.5	259	100
0.01	4.5	260	100
0	5.5	203	77
0.10	5.5	251	97

results of a previous investigation conducted in this laboratory concerning the effect of neutral salts upon purified preparations of malt amylase.[21] The enzyme preparations and starch samples which had served for the previous research some fifteen years ago and those made for this present study were employed for these comparisons. The essential difference between the earlier and the present procedures lay in the adjustment of the substrate. In one case the starch was "neutralized" with sodium hydroxide using rosolic acid as an indicator which would give solutions of about pH6.0, while in this investigation the reaction of the solutions was more definitely known and controlled by the use of acetate-acetic acid mixtures. Direct comparisons of enzymic activity were therefore made with malt amyalse acting upon (a) 2% starch which contained enough 0.01 M sodium hydroxide to be neutral to rosolic acid and (b) 2% starch adjusted by means of 0.01 molar acetate to approximately the same hydrogen ion activity. These activities were compared with that shown by the enzyme when acting upon 2% starch adjusted to a pH value of 4.5 by 0.01 molar acetate. As always the hydrogen ion activities of all substrate samples were measured electrometrically. In the three separate comparisons which were made with the

different amylase preparations under the above conditions (pH=6.0), malt amylase exerted higher activity in the presence of 0.01 or 0.02 molar sodium chloride than in its absence but this increased activity at pH6.0 did not even remotely approach that of the enzyme when acting in solutions adjusted to the optimal value of pH 4.5.

These results confirm those reported by Sherman and Thomas[21] in that the presence of sodium chloride aids the enzyme action in so-called neutral starch whose reaction corresponds to a pH value of about 6.0. The present work extends the former contributions in showing that the influence of sodium chloride upon purified malt amylase preparations should not be termed activation because the enzymic activity even with the addition of the optimal amount of sodium chloride in these more alkaline solutions does not reach that exerted by the enzyme at its optimum in the absence of this neutral salt. The advantages of a buffered substrate for the study of the amylases as well as the importance of establishing the best conditions for enzyme action are thus emphasized, and one reason for many of the seemingly contradictory findings of different workers in this field is apparent.

Results with Potassium Chloride, Sodium Sulfate and Sodium Nitrate. Comparisons of the influence of potassium chloride, sodium sulfate and sodium nitrate in 0.01 molar concentration upon the activity of malt amylase gave results of a nature similar to those obtained with sodium chloride. Consequently it did not seem necessary to determine the enzymic activity at closely graded intervals of hydrogen ion activity in the presence of each of these salts. Direct comparisons of the effect of these salts in 0.01 molar concentration upon malt amylase were made at the following pH values: 4.0, 4.3, 4.5, 5.0, 5.5. The point of reference chosen for these comparisons was the activity of malt amylase at pH=4.5 in the absence of salt. The effect of higher concentrations of these salts was also determined at pH values of 4.0, 4.5, 5.5. A few compari-

sons of the influence of these salts made upon one of the older preparations of the amylase yielded the same general effects but smaller differences in enzymic activity were obtained.

Results with Potassium Chloride. Three different samples of potassium chloride which had been purified either by recrystallization or by precipitation by means of hydrogen chloride were compared as to their influence upon the activity of different malt amylase preparations and were found to give identical results.

Potassium chloride in 0.01 molar concentration was found to aid the hydrolysis of starch by malt amylase in solutions more or less acid than the optimum. Average differences in enzymic activity corresponding to 25, 2, 4, 7, and 19 milligrams cuprous oxide at pH values of 4.0, 4.3, 4.5, 5.0 and 5.5 respectively indicate that the activity curve of malt amylase in the presence of 0.01 molar potassium chloride would resemble that of the enzyme in the presence of 0.01 molar sodium chloride which is shown in Figure I. Not only does 0.01 molar potassium chloride help the amylase action at unfavorable hydrogen ion activities but identical average differences in enzymic activity were obtained due to the presence of these two chlorides in 0.01 molar concentration in solutions of the pH values of 4.0 and 5.5.

When the concentration of potassium chloride was increased from 0.01 molar to 0.10 molar, malt amylase was affected in a similar manner as with sodium chloride. This is illustrated by the typical comparisons given in Table V.

TABLE V

INFLUENCE OF THE CONCENTRATION OF POTASSIUM CHLORIDE UPON THE ACTIVITY OF MALT AMYLASE

(Typical Individual Comparisons)

pH	4.0		4.5		5.5	
Molar Conc.	Activity	Increased Activity due to salt	Activity	Increased Activity due to salt	Activity	Increased Activity due to salt
K Cl	mg. Cu_2O		mg. Cu_2O		mg. Cu_2O	
0	230		258		182	
0.01	253	23	260	2	209	27
0.05	250	20	267	9	234	52
0.10	246	16	266	8	232	50

At pH=4.0 malt amylase was most active in the presence of 0.01 molar potassium chloride. With addition of the salt up to 0.10 molar concentration no significant change in activity occurred although a slight but apparently negligible decrease was noticed. In the region of the optimal hydrogen ion activity of malt amylase (pH=4.5) there appeared to be a tendency toward an increased activity of the enzyme with increasing concentration of the salt, of a slight activating influence. Potassium chloride resembled sodium chloride in that its favorable influence upon the activity of malt amylase at pH 5.5 was not complete until present in 0.05 molar concentration, above which an increase to 0.10 molar potassium chloride did not enhance the action of the enzyme.

That very similar results have been obtained for the effect of higher concentrations of sodium and potassium chlorides upon malt amylase at pH values of 4.0 and 5.5 is clear from the data in Table VI.

TABLE VI

COMPARISON OF THE CONCENTRATION EFFECT OF SODIUM AND POTASSIUM CHLORIDES UPON THE ACTIVITY OF MALT AMYLASE

(Average Increase in Activity due to Salt)

pH	4.0		4.5		5.5	
Salt	NaCl	KCl	NaCl	KCl	NaCl	KCl
Molar Conc.	mg. Cu_2O		mg. Cu_2O		mg. Cu_2O	
0.01	24	23	4	5	23	27
0.05	21	20	6	12	44	52
0.10	18	16	5	10	48	50

Although malt amylase appears to be favored at pH 4.5 very slightly by the potassium salt, differences in the influence of the two chlorides under these conditions do not appear to be decisive. Any favorable influence of the potassium ion would presumably be more noticeable under the less favorable conditions at pH 4.0 and pH 5.5, which is not the case. Thus the sodium and potassium ions appear to have an equal effect upon the hydrolysis of starch by malt amylase.

Results with Sodium Sulfate. In the presence of 0.01 molar sodium sulfate malt amylase was more active at the unfavorable hydrogen ion activity of pH 4.0 than in the absence of sulfate, but the average increase in enzymic activity, 13 milligrams cuprous oxide, was only about one-half that caused by the chlorides under the same conditions. In solutions of pH 4.3 and 4.5 the enzyme was not appreciably affected by 0.01 molar sodium sulfate. In the more alkaline solutions of pH 5.0 and 5.5 a small increase in enzymic activity corresponding to about 10 milligrams cuprous oxide was noted.

Higher concentrations of sodium sulfate up to 0.10

molar were found to show a small unfavorable effect at pH 4.0 upon the enzymic activity. A small increase in activity corresponding to 10 milligrams cuprous oxide was found between the activity of malt amylase at pH 4.5 in the presence of 0.01 and 0.10 molar salt. In this respect sodium sulfate resembles potassium chloride. In view of the fact that the beneficial influence of sodium sulfate upon the enzyme at unfavorable hydrogen ion activities was less than that of sodium and potassium chlorides, it seems unlikely that this small difference in amylase activity at pH 4.5 indicates an activating influence of the sulfate ion. At pH=5.5 an increase in the concentration of sodium sulfate up to 0.05 molar caused a decided rise in enzymic activity which was not exceeded as the salt concentration was increased to 0.10 molar. This increase of 30 milligrams cuprous oxide was equivalent to about seven-tenths of that shown by the chlorides at the same concentration and hydrogen ion activity.

Results with Sodium Nitrate. Sodium nitrate in 0.01 molar concentration gave evidence of a favorable influence upon the activity of malt amylase in solutions more acid than the optimum. The increase in enzymic activity was of the same magnitude as that caused by the same concentration of sulfate under the same conditions (pH= 4.0). In solutions of the other hydrogen ion activities investigated pH=4.3, 4.5, 5.0, 5.5) the enzyme was not affected by this salt in 0.01 molar concentration.

Higher concentrations of sodium nitrate up to 0.10 molar were found to result in a decidedly harmful effect upon the activity of malt amylase on the acid side of the optimum at pH 4.0 and in a very slight beneficial effect on the alkaline side of the optimum at pH 5.5. The differences in the enzymic activity due to the presence of 0.10 molar sodium nitrate under these conditions correspond to —25 milligrams and +10 milligrams cuprous oxide respectively. With a hydrogen ion activity of pH 4.5 a slight decrease in the amylase action was obtained with increasing concentrations of sodium nitrate.

Summary and Discussion

In order that sodium chloride, potassium chloride, sodium sulfate and sodium nitrate might be compared with respect to their influence upon the activity of malt amylase, all data for the enzymic activity in the presence and absence of each salt have been averaged. Single direct comparisons of the action of the enzyme in the presence of two or more separate salts accord with and thus warrant these averages.

In 0.01 molar concentration the chlorides of sodium and potassium show the most pronounced influence upon the enzymic action in solutions adjusted to hydrogen ion activities on both sides of the optimum. Nitrate and sulfate favor the starch hydrolysis to about one-half the extent caused by the chlorides at pH 4.0. At pH 5.5 nitrate in this concentration does not influence the action of malt amylase whereas sulfate does give evidence of a small favorable influence, about one-third that shown by the chlorides.

The best conditions for enzymic action constitute a better basis for comparison. Accordingly the influence of the four salts in 0.01 molar concentration at pH 4.0 and in 0.10 molar concentration at pH 5.5 may well be compared, for these salt concentrations were found, within the limits studied, to be optimal for the action of malt amylase at these pH values. It is noteworthy that when the hydrogen ion activity of the substrate is especially unfavorable (pH=5.5) a higher concentration of neutral salt is necessary for its most favorable action on the enzyme. The amount and the percentage increase in enzymic activity are greater at pH 5.5 than at pH 4.0 except in the case of nitrate. To ascertain to what extent the deleterious influence of the unfavorable hydrogen ion activities corresponding to pH values of 4.0 and 5.5 is overcome by the presence of salts in optimal concentra-

tion, the average activities of the enzyme under these conditions have been compared with the average activity of malt amylase at pH 4.5 in the absence of neutral salt. The two chlorides in solutions more or less acid than the optimum restore the enzyme almost wholly to its highest activity. In the presence of the most favorable concentration of sodium sulfate, the activity of malt amylase at pH 4.0 and 5.5 is the same percentage of the highest value of enzymic activity in the absence of salt. Sodium sulfate fails to restore the enzyme to its highest activity by five percent. In the case of nitrate the enzyme is influenced much more advantageously in solutions on the acid side of the optimum where nitrate causes the same degree of increase as sulfafe. On the alkaline side of the optimum, nitrate exerts a less pronounced effect upon the activity of malt amylase.

Comparison of the Effect of Neutral Salts Upon Pancreatic and Malt Amylases. A detailed study of the interrelation of neutral salts and hydrogen ion activity upon the hydrolysis of starch by pancreatic amylase has been made in this laboratory.[34] The results of the present investigation regarding malt amylase may well be compared with the data concerning pancreatic amylase since the experimental conditions under which the enzymes hydrolysed starch were the same or strictly comparable. The hydrogen ion activity of the subtrate was adjusted by 0.01 molar phosphate in the case of pancreatic amylase, while 0.01 molar acetate, which is a more suitable buffer for malt amylase[40] was used in this study to control the reaction of the solutions. Neither of these salts influence the enzymic activity under the conditions used. The same samples of sodium sulfate, sodium nitrate and potassium chloride were used in the two investigations.

The influence of neutral salts upon these two enzymes differs in that the presence of some neutral salt is essential for the activity of pancreatic amylase, whereas malt amylase remains active upon the removal of all neutral salts. Thus while neutral salts actually activate pan-

creatic amylase, they have been found in this present study only to favor the activity of malt amylase in solutions of unfavorable hydrogen ion activities. Sherman, Caldwell and Adams[34] found that the chloride ion caused the greatest activation of pancreatic amylase. In the case of malt amylase as well, the chloride ion increases the enzymic activity more than the other two anions investigated. The actual difference in enzymic activity due to the presence of chloride ion was much greater in the case of pancreatic amylase than with malt amylase. The activation of the former amounted to six to nine times the increase in enzymic activity afforded the latter. Malt amylase appears to be much less sensitive to the influence of neutral salts. Moreover there was no appreciable difference in the effect of the chlorides of sodium and potassium upon either of these two amylases.

Another similarity in the behavior of pancreatic and malt amylases exists in that they are affected less by the nitrate ion than by the chloride ion. They differ in their response to the sulfate ion, which has no influence upon pancreatic amylase but affects malt amylase in a manner similar to that exerted by the chloride ion, but to a smaller degree. The effect of neutral salts upon the activity of each enzyme appears to be due mainly to the anions and to be dependent upon the individual salt rather than upon its valence type. With both amylases the influence of equal concentrations of nitrate and chloride under similar conditions differed widely. That these univalent anions at the low concentrations here considered did not exert the same effect upon the starch hydrolysis would indicate that the influence of these salts upon the systems is not a matter of the total ionic strength and activity of the solutions.

The nature of the relation between neutral salts and amylase action has not yet been satisfactorily explained.[43] The coincidence of the isoelectric zone and the zone of optimal activity for malt amylase as established by Sherman, Thomas and Caldwell[24] indicates that "the enzyme exerts

its optimum effect upon starch where it is in the least ionized and least hydrated condition." That neutral salts increase the activity of malt amylase in solutions of hydrogen ion activities which are somewhat removed from the optimum may be due to a slight dehydrating effect of the salts upon the enzyme, whereby it attains a state more like that existing at the optimum. Higher concentrations of salts might in turn be expected to decrease the activity of the enzyme, if the particles were dehydrated to such an extent that coalescence and consequent decrease in the total surface of the enzyme resulted.

Purity of Materials. That some impurity or impurities had not been removed from two samples of sodium chloride during the usual purification processes was evident when malt amylase was found to be inactivated and to a different degree in the presence of 0.01 molar concentrations of these samples of sodium chloride. The sensitiveness of enzymes to very small concentrations of certain substances not only necessitates great care in experimentation with them but also explains why so many contradictory statements concerning them may be found in the literature. The necessity of preparing sodium chloride of the utmost purity obtainable was met by adapting the procedures advocated by Richards and Wells.[37] The method of purification has been described. That the impurity or impurities had been successfully removed was deducted from the fact that the purified sodium chloride did not inactivate malt amylase. The failure of different concentrations of this salt from 0.01 to 0.10 molar to affect the enzyme at the optimum indicated that no impurity which influenced the enzyme was present.

When the materials employed in this investigation were subjected to spectrographic examination through the courtesy of Professor H. T. Beans and Dr. A. Dingwall, they were all found to be of a high degree of purity. One of the samples of sodium chloride which had been purified by precipitation by hydrogen chloride but which had inactivated malt amylase showed the absence of all

foreign metals including iron except for a possible trace of aluminium. The influence of very dilute aluminium sulfate corresponding to the amount of aluminium which was estimated to be present in this sample of sodium chloride was measured upon the activity of malt amylase at pH=4.5. No change in enzymic activity was noted. Since suspensions of aluminium hydroxide have been used in adsorption methods for the purification of malt amylase it would be surprising to find that the aluminium ion in dilute solutions could exert a harmful influence upon the enzyme. Spectrograms were also made of sodium chloride which had been obtained by Richard's method, potassium chloride, sodium acetate, acetic acid and the purified preparation of malt amylase. Traces of sodium and aluminium were found in the potassium chloride. The similar influence of the chlorides of sodium and potassium upon the activity of malt amylase would indicate that a trace of sodium in the potassium salt would not be harmful. The enzyme preparation was found to contain an appreciable amount of calcium as well as a very small trace of magnesium. Since the period of dialysis of malt extract was sufficient to allow the complete removal of sodium and potassium, it is possible that the calcium remaining in the purified preparation of malt amylase may be in combination. Iron, manganese and zinc as well as sodium and potassium were not contained in this enzyme preparation. Although the spectrograms did not show the factor causing the difference in the influence of two of the samples of sodium chloride upon the activity of malt amylase, nevertheless they served to prove the absence of certain suspected impurities such as iron, and to show the high degree of purity attained in the purification procedures followed in this laboratory.

Conclusions

The influence of sodium chloride, potassium chloride, sodium sulfate and sodium nitrate upon the saccharogenic activity of malt amylase has been investigated and special consideration has been given to the influence of changes in hydrogen ion activity and salt concentration.

Neutral salts are not essential to the activity of malt amylase nor do they increase the saccharifying action of the enzyme above that shown in the absence of neutral salt at the optimal hydrogen ion activity.

The activity of malt amylase is favored at unfavorable hydrogen ion activities by the presence of neutral salts.

The optimal concentrations of sodium chloride for the activity of malt amylase at pH 4.0 are 0.01 molar and at pH 5.5 are 0.05 to 0.10 molar. Within the limits here considered, the more unfavorable the hydrogen ion activity (pH=5.5), the higher the concentration of salt required for its most favorable action on the enzyme.

The addition of sodium chloride in concentrations most favorable to the action of malt amylase at pH values of 4.0 and 5.5 restores the enzyme to an activity equal to the highest activity obtaining in the absence of the salt at pH 4.5. Thus with the addition of the proper amount of sodium chloride malt amylase is able to exert its highest activity in solutions differing more widely in hydrogen ion activity than in its absence.

Potassium chloride is equal to, if not slightly more favorable than sodium chloride in its influence upon the activity of malt amylase under like experimental conditions.

Sodium sulfate and sodium nitrate also increase the activity of malt amylase at unfavorable hydrogen ion activities but to a smaller degree than the chlorides.

The influence of neutral salts upon the activity of malt amylase appears to be due mainly to the anions and to be specific.

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